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14. ABSTRACT

The objective of this study is to develop a protein that can be injected directly into the eye while in the combat theater to prevent or reduce the damage to retinal cells following blast associated trauma. To this end, we propose use of a peptide (POD, peptide for ocular delivery) that we have previously shown to be capable of penetrating retinal cells in vivo. POD will be utilized to deliver melanoma inhibitor of apoptosis (ML-IAP) or X-linked inhibitor of apoptosis (XIAP) into retinal cells, neither of which have a cell penetrating capacity by themselves. We are using recombinant adenovirus as the gene expression system to synthesize the POD fusion proteins. At the end of Year 1 of this proposal, we have completed the construction of 1 (XIAP) of the recombinant adenovirus constructs and are at the penultimate step of generating the ML-IAP construct. Next steps are to purify these two proteins and evaluate their anti-apoptotic properties in vitro and ultimately in vivo. During the following year we will complete the construction of the ML-IAP construct and relevant controls and perform studies to test the anti-apoptotic properties of these recombinant proteins.

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Platform for Rapid Delivery of Biologics and Drugs to Ocular Cells and Tissues Following Combat Associated Trauma

ANNUAL REPORT 2013

INTRODUCTION:

Blast injury induces several cell death pathways including apoptosis [1]. Retinal ganglion cells and photoreceptors are not capable of self-renewal and loss of retinal neurons leads to impaired vision or complete blindness. It is imperative to block cell death pathways such as apoptosis subsequent to acute trauma as soon as possible, ideally by self-administration of a drug or a biologic that can be included in the medical kit. Developing a platform technology and proving its usefulness in at least one pathway, i.e. apoptosis, is the major goal of this proposal. The peptide for ocular delivery (POD) has been validated for efficient transport of DNA and proteins to the eye [2-5]. Since DNA expression is slow, our goal is to develop a protein that could act immediately upon injection prior to the onslaught of the damage created by apoptosis. We envisage a lyophilized product provided in the medical kit that the member of the armed forces could re-suspend in packaging and request a colleague to inject into the eye or self administrate if necessary. In this study, several different adenovirus constructs will be developed to generate recombinant protein - POD fusions with the anti-apoptotic proteins 'melanoma inhibitor of apoptosis (ML-IAP) and X-linked inhibitor of apoptosis (XIAP; see supplemental Figure 1). The purified proteins will then be evaluated in vitro and in vivo for their anti-apoptotic and thereby protective capacities. We will utilize a blue light induced model of apoptosis in mice, a model system that has been well characterized in our laboratory and intended to act as a surrogate for trauma associated with any event that triggers apoptosis such as blast wind, radiation, heat etc.

BODY:

1. Production of DNA constructs for production of adenoviruses

The first step of generating purified recombinant POD-fusion proteins is to generate several DNA expression cassettes in a human recombinant adenovirus expression system. To this end, bacterial clones carrying plasmids that contain the gene sequences for the human melanoma inhibitor of apoptosis protein (ML-IAP) and the X-linked inhibitor of apoptosis protein (XIAP) [IMAGE clones 4859588 and 5532247] were purchased from the American Type Culture Collection (ATCC).

The plasmids were purified and used as templates for a PCR amplification of the open reading frames (ORF) with gene specific primer pairs. These were designed such that the cDNAs could be inserted in the POD-GFP delivery vector pQBI25-fA1-POD-GFP and replace the GFP part by the IAP sequence. Cloning details available upon request. The plasmids for the corresponding controls (IAPs without POD) were constructed by a similar strategy. Finally, 6x histidine (his) tagged POD-MLIAP

and POD-XIAP fusion genes and 6x his tagged MLIAP an XIAP genes regulated by the strong cytomegalovirus (CMV) promoter were created as a first step:

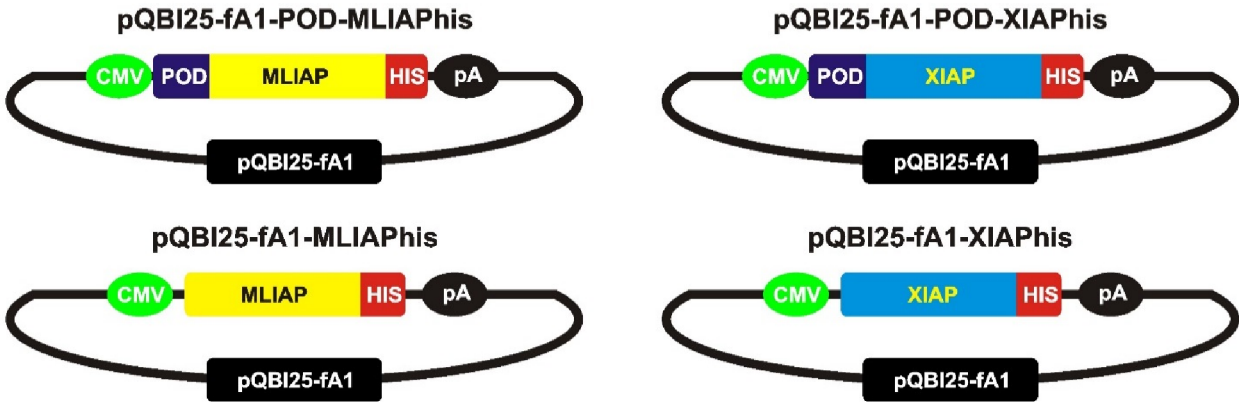


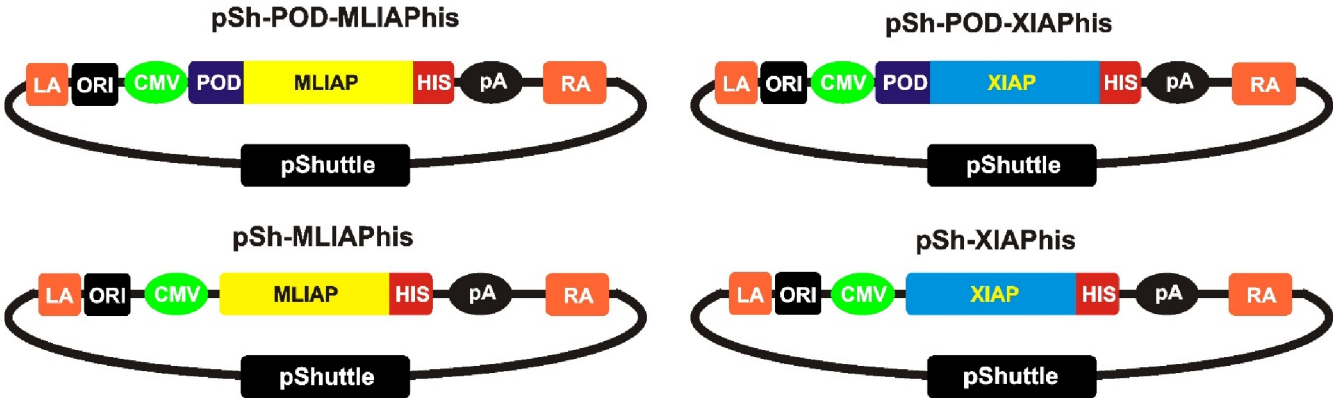
Figure 1: Expression plasmids for POD-IAP fusions and corresponding controls.

All four plasmids were sequenced to exclude PCR mutations and the authenticity of the constructs was confirmed. Functionality of the constructs with respect to protein synthesis was confirmed in western blot analyses by detection of the fusion proteins with his-tag-specific antibodies in whole cell extracts of transfected 911 cells (shown for the POD-fusion plasmids in Figure 2B).

In the second cloning step, the entire expression cassettes (CMV promoter, coding sequence, poly adenylation signal) of both clones were transferred into the vector pShuttle (Figure 2A).

The resulting plasmids were required to produce genomes of POD-ML-IAP-his and POD-XIAP-his adenoviruses by subsequent homologues recombination. As for the pQBI-plasmids, functionality of the pShuttle-plasmids was confirmed by western blot analyses of protein extracts of transfected 911 cells (shown for the POD-fusion plasmids in Figure 2B).

A



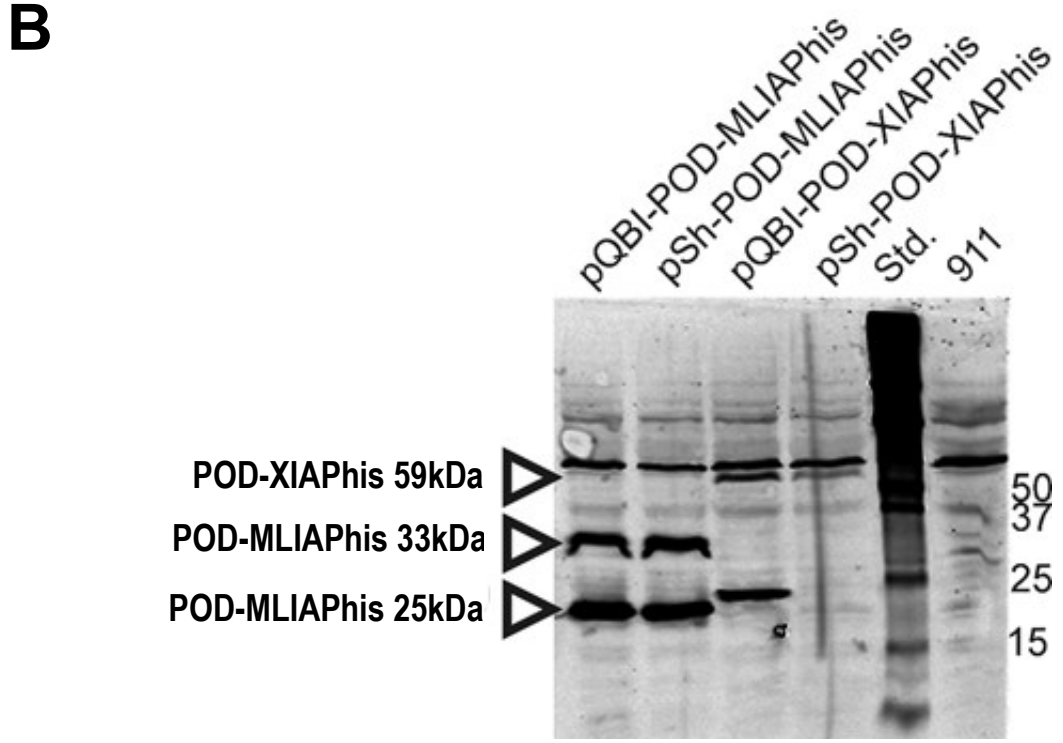


Figure 2: Shuttle vectors (A) and Western blot analysis of protein expression (B)
(**A**) Graphical schemes of the constructed shuttle plasmids. (**B**) Western blot analysis demonstrates expression of both POD-IAP fusion proteins after transfection of the pQBI25fA1 plasmids (lanes 1 and 3) and the pShuttle plasmids (lanes 2 and 4). POD-XIAPhis is detected as a single band at 59kD (lanes 3, 4), which is absent in 911 cells transfected with MLIAP-plasmids (lanes 1, 2) and untransfected 911 cells (lane 6). POD-MLIAPhis is detected by two bands at 33kD and 25kD, which are absent in 911 cells transfected with XIAP-plasmids (lanes 3, 4) and untransfected 911 cells (lane 6).

2. Production and selection of recombinants

As indicated above, the process of generating adenoviral genomes is based on homologous recombination between the shuttle plasmids and the vector pAdEasy-1 containing the virus components. Therefore, linearized shuttle plasmids (*PmeI*-digested) were transformed into the bacterial strain BJ5183-AD-1 (Stratagene) by electroporation. These bacteria are pre-transformed with AdEasy-1 and allow highly efficient recombination. Resulting recombinants were initially screened by PCR using AdEasy-1 and IAP specific primers. PCR-positives where then tested for correct recombination by elaborate restriction-digest analyses as only 100% proof clones qualify for the subsequent steps of adenovirus production.

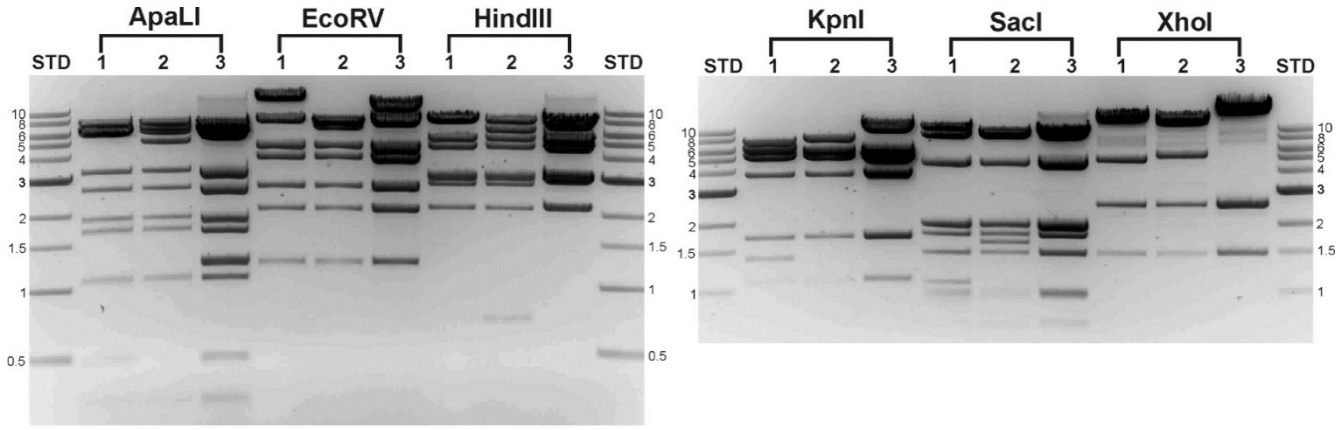


Figure 3: Example of a restriction-digest analysis
Restriction fragment patterns of pAd-1-MLIAPhis clone 5 (1) and pAd-1-XIAPhis clone 3 (2) digested with six different restriction endonucleases compared to the fragment pattern of the original pAdEasy-1 vector (3) confirming correct homologue recombination.

Two confirmed clones for each POD-fusion and one clone for each control were obtained: pAd-1-POD-MLIAPhis (clones 1, 3); pAd-1-POD-XIAPhis (clones 3, 4); pAd-1-MLIAPhis clone 5; pAd-1-XIAPhis clone 3.

In summary, step 1 of the adenovirus-based production of POD-IAP fusions and corresponding controls according to the definition in milestone 1 (*generation of adenovirus-genome-coding DNA-constructs*) was accomplished.

3. Production and purification of adenoviruses

At the first step of the adenovirus production the Ad-1 clones produced in quarters 1 and 2 were linearized (*PacI*-digested) and used to transfected 911 cells (60mm-plate scale). Seven days after transfection, cell cultures that showed signs of cytopathic effects (CPE; hallmark for virus production) were harvested and lysed by repeated freeze-thaw cycles to obtain 'crude adenovirus lysates'. These were used for subsequent infections of 911 cells (five 100mm-plates each lysate) to up-scale virus yield. To the end of quarter 4, only clones pAd-1-POD-MLIAPhis 3 and pAd-1-POD-XIAPhis 4 showed weak CPE and were further processed, i.e. used for the infection of 100mm plates. Seven days after infection, viruses were harvested, lysed by repeated freeze-thaw cycles and purified using the commercial Adenopure® purification kit. Aliquots were stored at -80°C. Purified viruses and crude lysates of the other clones obtained at earlier stages of the production process were tested for infectivity and protein expression (see next paragraph). Currently all of the clones are at the production stages, testing modifications of transfection and infection to increase success-rates.

4. Functionality testings

Viruses of POD-IAPhis fusions were used in first experiments testing functionality. Therefore ARPE-19 cells were infected at a multiplicity of infection (MOI) of 1000, cells harvested seven days after infection and protein contents isolated. Expression was tested by western blot as already described. First results indicate expression of the POD-MLIAPhis fusion after infection.

Crude lysates of the other clones were tested in infections of 911, 293 and ARPE-19 cells but efficacy was very low and protein expression was not detected. As mentioned, production of new batches started in quarter 3 which are currently being processed.

ISSUES:

In our initial attempt we failed to recover the desired recombinant clones but we have solved the technical issues and some of the desired clones have now been rescued. Overall, the project is in line with the schedule defined in the statement of work (SOW).

KEY RESEARCH ACCOMPLISHMENTS:

- DNA constructs coding for POD-IAP fusions and corresponding controls were generated
- pAd-1 x pSh recombinants were generated

CONCLUSION:

Aims defined in the SOW for year one of the project were accomplished. Production and purification of Adenoviruses is currently in progress, protein purification is expected to be started early 2014.

REFERENCES:

1. Svetlov SI, Larner SF, Kirk DR, Atkinson J, Hayes RL, and Wang KK: *Biomarkers of blast-induced neurotrauma: profiling molecular and cellular mechanisms of blast brain injury.* J Neurotrauma, 2009;26(6):913-21.

2. Johnson LN, Cashman SM, and Kumar-Singh R: *Cell-penetrating Peptide for Enhanced Delivery of Nucleic Acids and Drugs to Ocular Tissues Including Retina and Cornea*. Mol Ther, 2007;16(1):107-14.

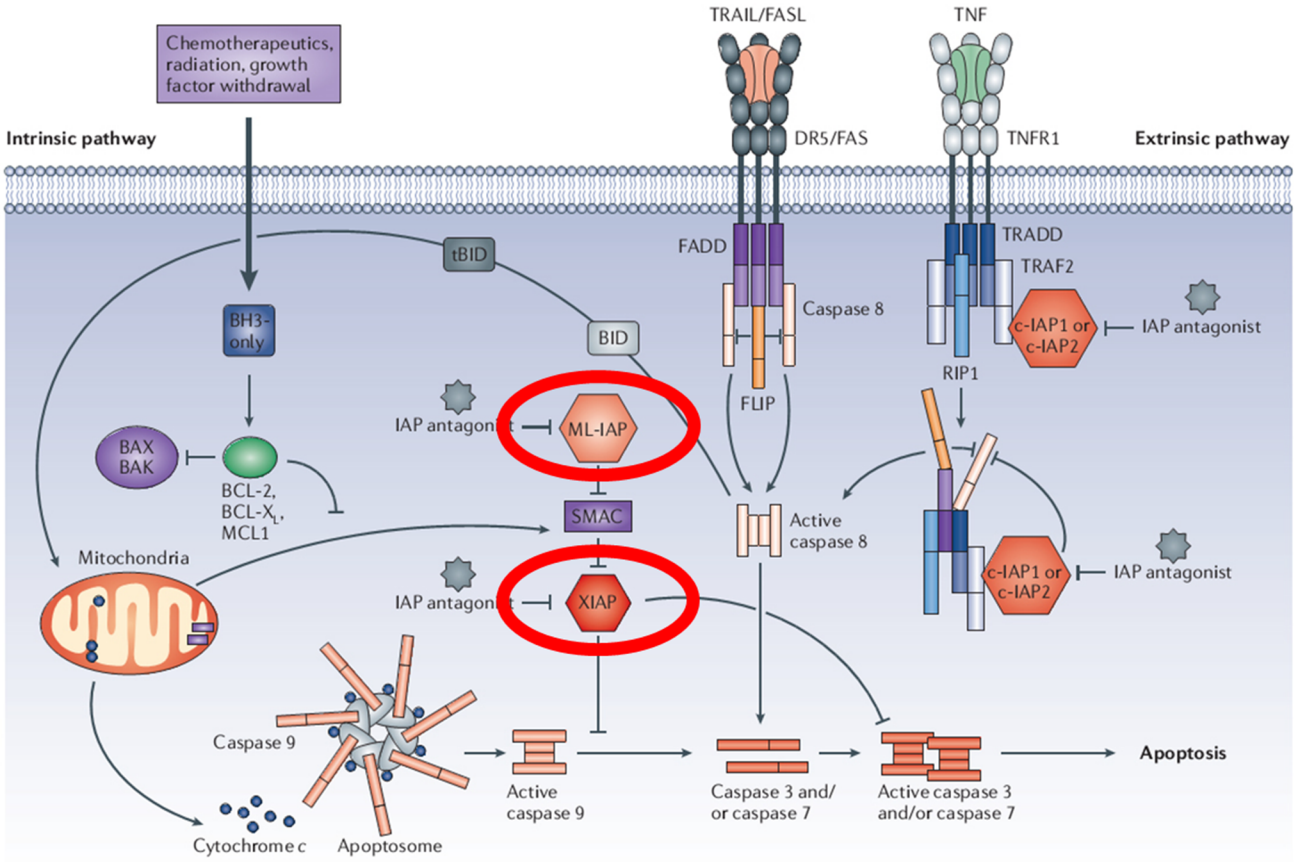
3. Read SP, Cashman SM, and Kumar-Singh R: *POD Nanoparticles Expressing GDNF Provide Structural and Functional Rescue of Light-induced Retinal Degeneration in an Adult Mouse.* Mol Ther, 2010;18(11):1917-26.

4. Read SP, Cashman SM, and Kumar-Singh R: *A poly(ethylene) glycolylated peptide for ocular delivery compacts DNA into nanoparticles for gene delivery to post-mitotic tissues in vivo*. J Gene Med, 2010;12(1):86-96.

5. Johnson LN, Cashman SM, Read SP, and Kumar-Singh R: *Cell penetrating peptide POD mediates delivery of recombinant proteins to retina, cornea and skin*. Vision Res, 2010;50(7):686-97.

6. Fulda S, and Vucic D: *Targeting IAP proteins for therapeutic intervention in cancer.* Nat Rev Drug Discov, 2012;11(2):109-24.

SUPPORTING DATA:



(modified from Ref. 6: Fulda S, and Vucic D: *Targeting IAP proteins for therapeutic intervention in cancer*. Nat Rev Drug Discov, 2012;11(2):109-24.)

Supporting Figure 1: Functions of MLIAP and XIAP in the process of apoptosis

XIAP inhibits the activation of pro-caspases 3 and 7 by caspase 9 in the intrinsic pathway of apoptosis. Moreover it blocks active caspases 3 and 7 activated by caspase 8 in the extrinsic pathway (FAS and TNF elicited). MLIAP blocks the XIAP inhibitor SMAC (DIABLO), which is released from mitochondria when the intrinsic pathway of apoptosis is activated.